CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmaceutical Chemistry and Drug Analysis

Analysis of chiral resorcin[4]arenes by enantioselective HPLC and circular dichroism

---

Alma Mater Studiorum - University of Bologna

Department of Pharmacy and Biotechnology

Diploma Thesis

Author: Martina Přecechtělová

Supervisors: Prof. Carlo Bertucci

RNDr. Milan Mokrý, CSc.

Hradec Králové 2015

KARLOVA UNIVERZITA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra farmaceutické chemie a kontroly léčiv

Analýza chirálních resorcin[4]arénů pomocí enantioselektivní HPLC a cirkulárního dichroismu

---

Alma Mater Studiorum - University of Bologna

Department of Pharmacy and Biotechnology

Diplomová práce

Autor práce:	Martina Přecechtělová
Vedoucí práce:	Prof. Carlo Bertucci
	RNDr. Milan Mokrý, CSc.

Hradec Králové 2015

“I declare the thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature.”

Martina Přecechtělová

Acknowledgements

I would like to thank to Professor Carlo Bertucci, from the Department of Pharmacy and Biotechnology, Alma Mater Studiorum – University of Bologna, for being my supervisor and for helping me with writing the thesis.

I would like to thank to RNDr. Milan Mokrý, CSc. for being my supervisor at Faculty of Pharmacy in Hradec Králové.

I would like to thank and I am grateful to Daniele Tedesco, Ph.D. for introducing me the work in laboratory, for being supportive during my research and for helping me with writing the thesis.

I also would like to thank to my family for supporting me during my studies.

Abstract

Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Pharmaceutical Chemistry and Drug Analysis

Alma Mater Studiorum - University of Bologna
Department of Pharmacy and Biotechnology

Author: Martina Přecechtělová
Supervisors: Prof. Carlo Bertucci
RNDr. Milan Mokrý, CSc.
Consultant: Daniele Tedesco, Ph.D.

Title of Diploma thesis:

Analysis of chiral resorcin[4]arenes by enantioselective HPLC and circular dichroism

Enantioselective HPLC methods are among the most widespread methods for the enantioseparation of racemic drugs. Enantioselective HPLC is useful not only for the ability to perform quantitative and qualitative evaluations of separated enantiomers, but also for allowing the coupling of other analytical techniques able to expand its utilization. In this thesis I will study the enantioseparation of resorcin[4]arenes by enantioselective HPLC hyphenated to electronic circular dichroism (ECD), with the aim of a complete stereochemical characterization of samples. Resorcin[4]arenes ***7e*** and ***5a*** are new synthesized molecules, which have potential to be appropriate building blocks for the construction of a large variety of supramolecular hosts, such as self-assembled capsules, cavitands and carcerands. Enantiomeric separation and subsequent stereochemical characterization of these resorcin[4]arenes will contribute to the understanding and further utilization of their advantageous chemical properties.

Abstrakt

Univerzita Karlova v Praze
Farmaceutická fakulta v Hradci Králové
Katedra farmaceutické chemie a kontroly léčiv

Alma Mater Studiorum - University of Bologna
Department of Pharmacy and Biotechnology

Autor: Martina Přecechtělová
Školitel: Prof. Carlo Bertucci
RNDr. Milan Mokrý, CSc.
Konzultant: Daniele Tedesco, Ph.D.

Název diplomové práce:

Analýza chirálních resorcin[4]arénů pomocí enantioselektivní HPLC a cirkulárního dichroismu

Enantioselektivní HPLC metoda je jednou z nejrozšířenějších metod pro enantioseparaci racemických léčiv. Je výhodná nejen pro svou schopnost kvantitativního a kvalitativního hodnocení separovaných složek, ale také umožňuje propojení s dalšími analytickými přístroji a tím rozšiřuje své využití. V této práci se budu zabývat enantioseparací resorcin[4]arénů pomocí enantioselektivní HPLC spojenou s elektronovým cirkulárním dichroismem (ECD), s cílem o kompletní stereochemickou charakterizaci vzorku. Resorcin[4]arény ***7e*** a ***5a*** jsou nově syntetizované molekuly, které mají potenciál stát se vhodnými stavebními bloky pro stavbu velké palety supramolekulárních hostitelů, jako jsou samoorganizující kapsuly, kavitandy a kancerandy. Enantiomerická separace a následná stereochemická charakterizace těchto resorcin[4]arénů přispěje k pochopení a dalšímu využití jejich výhodných chemických vlastností.

Contents

Introduction

Chiral drugs and other chiral chemical structures are classes of compounds with high scientific and pharmaceutical potential. It was found that single enantiomers of racemic drugs can have different therapeutic effect in human body. It may exhibit the opposite or toxic effects. An example of a chiral drug with strong differences in the therapeutic and toxic potential of its enantiomers is thalidomide. Its R-isomer has sedative, antiemetic and hypnotic effects, while its S-isomer is highly teratogenic. The need of drugs with enantiomeric purity increased significantly in the last decades.

Enantioselective separation techniques such as high performance liquid chromatography (HPLC) based on the use of chiral columns is one of the most progressive and very effective methods which improve enantiomeric excess of chiral compounds. The best method to investigate of the stereochemistry of molecules is enantioselective high-performance liquid chromatography hyphenated to electronic circular dichroism (HPLC-CD). Compounds ***7e*** (1,2,3,4,5,6,7-*O*-heptabenzoyl-*C*-undecylresorcin[4]arene) and ***5a*** (1,2,4,6-*O*-tetrabenzyl-*C*-undecylresorcin[4]arene) are chiral and composed of two enantiomers. They have the potential to become building blocks for the preparation of supramolecular hosts.

The aim of my work was to obtain a separation of compound ***7e*** and ***5a***, and collect one of the enantiomers of compound ***7e*** for further modification and characterization.

1 Theoretical part

1.1 Chirality

Many compounds exist in two isomers, which have a similar relationship to the one between left and right hands: they are the mirror image of each other. This property is called chirality; chiral compounds do not have a plane or centre of symmetry, and the isomers of a chiral molecule with mirror-image configurations are called enantiomers. Enantiomers have identical chemical and physical properties, except of the fact that they interact in a different way with asymmetric environments: for example, enantiomers display optical activity, since they are able to rotate the plane of polarized light by the same angle but toward opposite sides. An equal mixture of two enantiomers is called racemate or racemic mixture.[1, 2]

1.2 Chirality in pharmaceutical sciences

Many pharmaceutically important compounds are chiral; nowadays, many formulations occur as racemic mixtures. The need for developing single enantiomeric formulations as therapeutics rather than using racemic mixtures arises from the fact that enantiomers usually display different biological activities: one of the enantiomers might be more pharmacologically active or more toxic than the other; enantiomers might even be active on different targets or contribute to side effects without having a therapeutic activity.[3] Minimizing the time needed to obtain pure enantiomers is of paramount importance in drug discovery.[3] For this purpose, the demand for the development of separation analytical methods able to distinguish between enantiomers began to increase.

1.3 Enantioselective HPLC

The determination of the enantiomeric purity of chiral substances, therefore, is an important topic in analytical chemistry, due to the importance of chiral compounds and their employment in pharmaceutical industry. Enantiomeric purity is also a required parameter for comparative biological, pharmacological or toxicological evaluation.[3]

Analytical and preparative separation techniques can be used to investigate the enantiomeric proportions of compounds. In particular, high-performance liquid chromatography (HPLC) based on the use of chiral columns is very reliable and the most frequently used technique for enantiomeric excess determination.[3]

Nowadays, HPLC is one of the most advanced analytical methods which can be used for enantiomeric separation. It enables qualitative and quantitative evaluation of enantiomeric components. HPLC usually furnishes both enantiomers with high enantiomeric purity. HPLC based on the use of chiral columns is one of the method, which we can used for the enantioseparation of chiral molecules, such as resorcin[4]arenes. For this purpose, many chiral stationary phases (CSPs) were designed to provide a successful separation of enantiomers.[4]

1.4 Chromatographic parameters

Chromatographic separations are characterized by factors such as retention times, capacity factors, resolution and selectivity (Figure 1, Table 1).
The retention time (t_R) is the time required by the compound in order to elute from the column, which is measured from the instant the sample is injected inside the HPLC system to the instant at which the detector measures the maximum of peak height for that compound.[5]

The capacity factor (k) is the ratio of the amounts of the solute at the stationary and mobile phases within the analyte band inside the chromatographic column. It is usually calculated from the retention times as follows:

$$k = \frac{t_R - t_0}{t_0} \quad \text{(Eq. 1)}$$

where t_R is the retention time for the peak and t_0 is the void time, that is the time at which the solvent of the injected sample is detected. [6]

Resolution ($\mathrm{R_S}$) is a parameter defining the quality of separation of two peaks. It measures how well two peaks can be differentiated in a chromatographic separation.[6] The equation of resolution is:

$$\mathrm{R_S} = \frac{2[(\mathrm{t_R})_2 - (\mathrm{t_R})_1]}{\mathrm{W_1 + W_2}} \quad \text{(Eq. 2)}$$

where $(t_R)_1$ is the retention time for the first eluted peak, $(t_R)_2$ is the retention time for the second eluted peak, W_1 is the width of the first peak and W_2 is the width of the second peak. Resolution values equal to or greater than 1.5 between two peaks will ensure that the sample components are properly separated.[6]

Selectivity (α) is the ability of the chromatographic system to distinguish between sample components, and is defined as:

$$\alpha = \frac{\mathrm{k_2}}{\mathrm{k_1}} = \frac{(\mathrm{t_R})_2 - \mathrm{t_0}}{(\mathrm{t_R})_1 - \mathrm{t_0}} \quad \text{(Eq. 3)}$$

where k_1 and $(t_R)_1$ are the capacity factor and retention time for the first eluted peak, k_2 and $(t_R)_2$ are the capacity factor and retention time for the second eluted peak, and t_0 is the void time.[6]

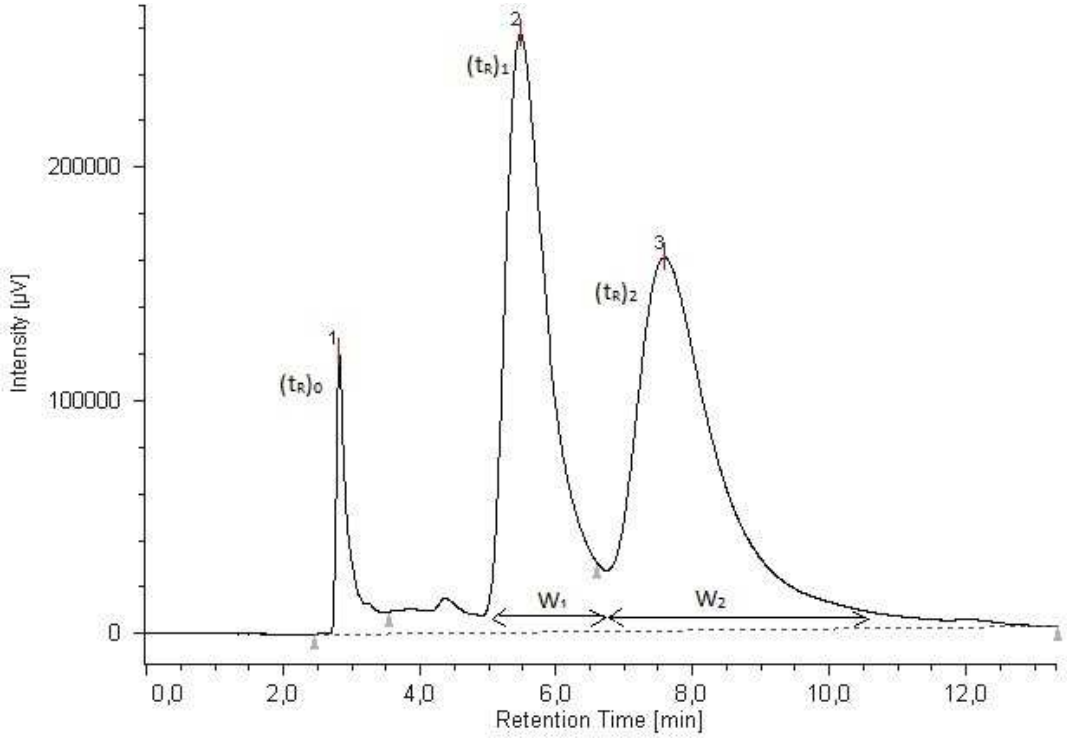


Figure 1. Retention times and width of peaks, shown on the chromatogram for the enantioselective HPLC analysis of compound ***7e*** on Chiralcel OD-H (mobile phase: *n*-hexane/2-propanol 97:3, v/v; flow rate: 1 mL/min; λ = 230 nm).

Table 1. Chromatographic parameters for the chromatogram shown in Figure 1.

Column	**Mobile phase**	**Composition (v/v)**	**Flow rate (mL/min)**	k_1	k_2	α	R_s
Chiralcel OD-H	*n*-hexane/2-propanol	97:3	1.0	0.938	1.694	1.806	1.371

Peaks between the peak of solvents and the peak of the first eluted fraction are undesirable. They are probably peaks of impurities or degradation products of the sample.

1.5 HPLC systems

The system for high-performance liquid chromatography (HPLC) is composed by a high pressure pump which is used for storing and transporting the mobile phase. The sample is loaded in the system by injecting a solution with a syringe inside the fixed-volume loop of an injection valve; rotating the valve from position LOAD to INJECT, the mobile phase starts to flow through the loop and transport the liquid sample into the system. The equipment providing the separation of the substance is the chromatographic column. The elution of analytes from the column can be obtained in isocratic (constant composition of the mobile phase) or gradient (changing composition of the mobile phase with time) conditions. A thermostat can be used to ensure constant temperature for the separation; finally, a detector is connecting to the column and controlled by a computer. The most commonly used detector is a UV-VIS detector; we also used this detector for my analyses.[7, 8]

1.6 Strategies for enantioselective HPLC separation

The formation of diastereomers, which allows separation of the enantiomers, can be obtained with two types of strategy - direct or indirect. In direct separation, a transit diastereomer is formed between the CSP (or chiral additive in mobile phase) and the enantiomer. In indirect separation, the diastereomers are formed by a chemical reaction between enantiomerically pure chiral reagents and the enantiomers of the analyte before injection. This reaction is performed in achiral environment. The direct enantioselective chromatography on CSPs is the most frequently used in drug discovery.[9] The most commonly available CSPs are based on:

- Macrocyclic glycoproteins;
- Polysaccharides;
- Crownethers;

- Cyclodextrines;
- Glycoproteins CSPs;
- Ligand exchange.

For this work, a direct method with CSPs based on polysaccharides was used.

1.7 Polysaccharide-based chiral stationary phases

Polysaccharides, such as cellulose and amylose (Figure 2), have an asymmetric structure, which is perfectly defined, and these molecules are among the most widespread optically active biopolymers, therefore they have a potential to be applied for chiral separation in liquid chromatography.[10-13]

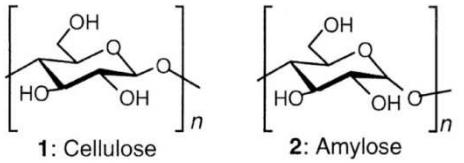


Figure 2. Structures of cellulose and amylose.[10]

Cellulose is a polymer composed of regularly repeating units of D-(+)glucose connected by β-1,4 glycosidic binding, while amylose is composed by regularly repeating units of D-(+)glucose connected by α-1,4 glycosidic binding. Native polysaccharides cannot be used as chiral stationary phases (CSPs) due to their low enantioselectivities and chemical properties. However, functionalized polysaccharides such as polysaccharides esters - cellulose triacetate or cellulose tribenzoates, and triphenylcarbamates (Figure 3) show high chromatographic resolution of a variety of racemates. CSPs are composed of functionalized polysaccharides, which can be immobilized or coated on a support such as silica gel.[11]

Different enantioselectivities are achieved with the different types of polysaccharide-based CSPs, due to the different conformations of amylose (helical) and cellulose (linear).[2]

The prediction of the most appropriate columns for enantiomeric separation is impossible; it depends on the chemical structure and geometric arrangement of the compound, and on the composition of the mobile phase.[2]

Often, only one functional group of the molecule is sufficient for the successful separation. In other situations, a defined geometrical conformation is needed to achieve a separation of enantiomers.[2]

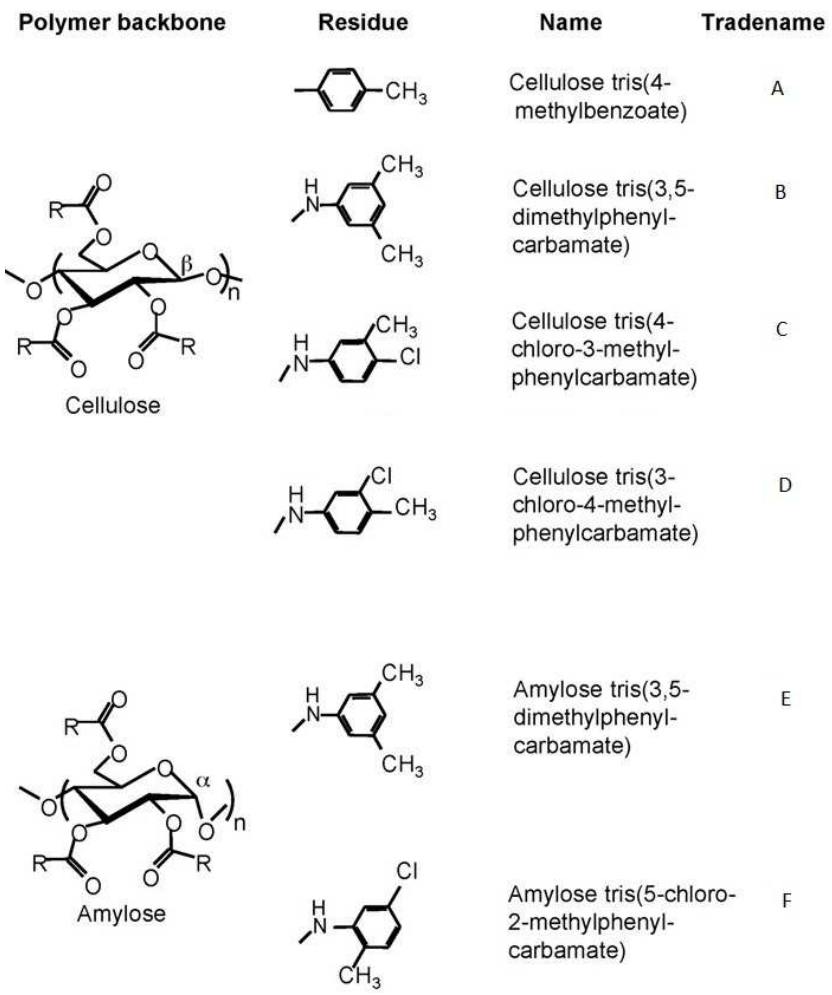


Figure 3. Selector structures of coated polysaccharide CSPs along with column trade names. A: Lux Cellulose-3, Chiralcel OJ; B: Lux Celulose-1, Chiralcel OD-H; C: Lux Cellulose-4; D: Lux Cellulose-2; E: Chiralpak AD; F: Lux Amylose-2. Chiralcel and Chiralpak are trade names of Daicel (Chiral Technologies), Lux of Phenomenex (Redesigned from ref.[4]).

1.8 Effect of mobile phase on enantioresolution

The mobile phase is the one of the most important factors for the enantioseparation of a racemate. Selectivity of the separation, retention time and resolution are very sensitive to changes in the composition of the mobile phase.[14]

In normal-phase chromatographic conditions, composition of polar and non-polar modifier influences the length of the retention time. “Normal-phase conditions” means that the non-polar mobile phase component is predominant over the polar component. Reduction of the retention time is usually achieved by increasing of proportion of the polar modifier (Figure 4) for polysaccharide-based CSPs, alcohols such as methanol, ethanol and 2-propanol are the most commonly used polar modifiers.[14]

Composition of the mobile phase may not only influence retention times, but it can also considerably affect selectivity (Figure 4). Each compound is individual, so we have to find the optimal parameters of the mobile phase for each compound separately. It is necessary to have an analyzed sample soluble in the mixture used as the mobile phase.[14]

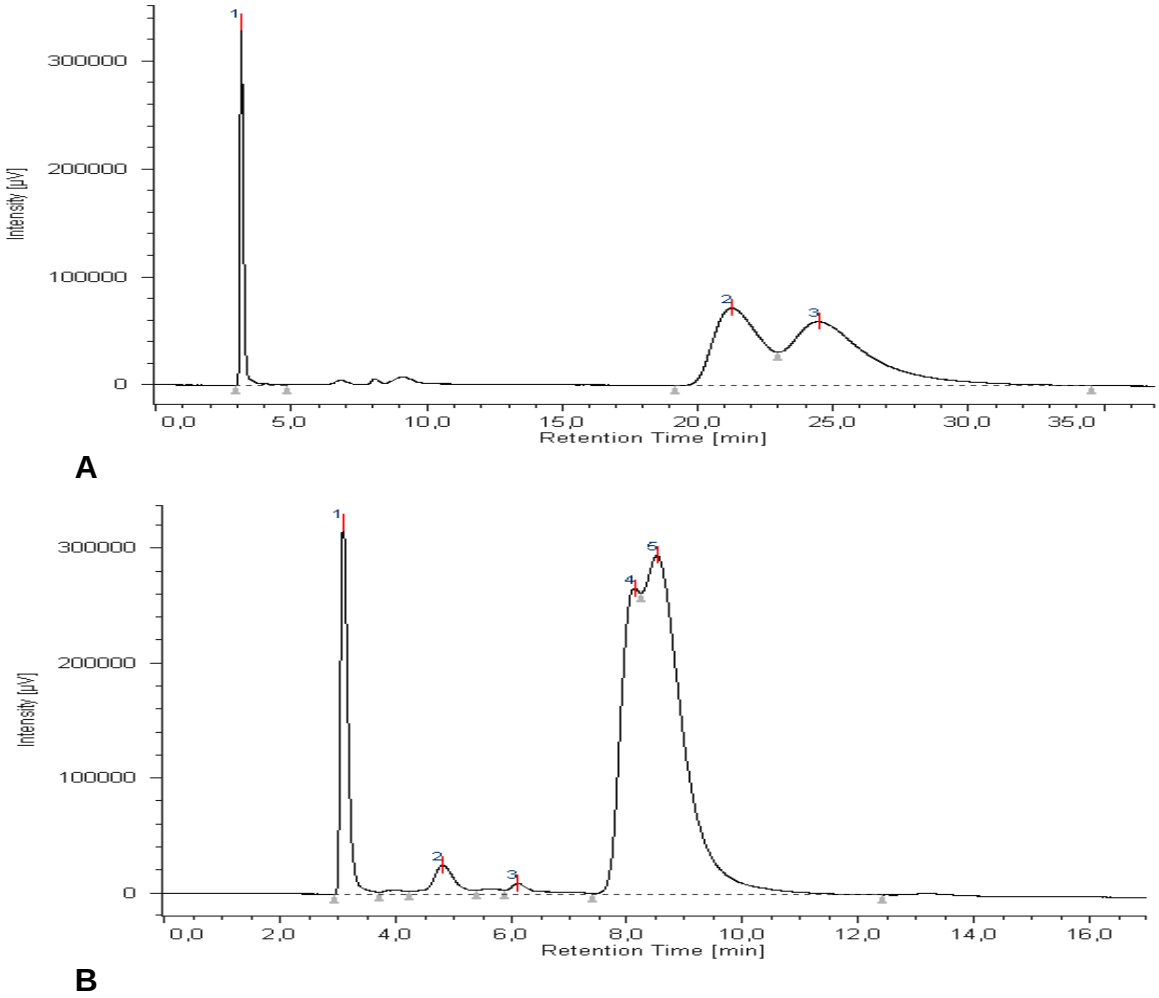


Figure 4. Effect of the proportion of polar modifier on enantioresolution. A: chromatogram for enantioselective HPLC analysis on ***7e*** on Chiralpak AD (mobile phase: *n*-hexane/2-propanol 99:1, v/v; flow rate: 1 mL/min; λ = 230 nm); B: chromatogram for enantioselective HPLC analysis on ***7e*** on Chiralpak AD (mobile phase: *n*-hexane/2-propanol 98:2, v/v; flow rate: 1 mL/min; λ = 230 nm).

For normal-phase conditions, it means to have a sample soluble in non-polar phase. If a sample is soluble in aqueous medium, we have two possibilities for HPLC analyses:

- Developing a method in polar organic mode;
- Developing a method in reversed-phase (RP) conditions.

The solvents used for mobile phases in polar organic mode are usually alcohols, acetonitrile and their combinations. However, for certain molecules, this strategy will not be sufficient, because the enantioselectivity in this mode is very limited. For this case, we can use the RP conditions. The mobile phase for RP mode is based on using a mixture of water and organic solvent, such as acetonitrile, 2-propanol or methanol. For some polar molecules, the presence of water in the mobile phase can have an impact for the enantioselectivity and offer a possibility of success for the resolution of enantiomers.[14]

1.9 HPLC-CD technique

The absolute configuration and enantiomeric excess are important factors to assess the stereochemistry of the chiral molecules. In order to obtain information about the absolute configuration (AC) of new types of chiral structures, HPLC separation methods may take advantage of the coupling with spectroscopic methods based on polarized light, like electronic circular dichroism (ECD). ECD spectroscopy can be used as a detection system for HPLC if the analyte absorbs radiation in the UV/VIS region and the relevant chromophore is close to a chiral center.[15]

Therefore one of the best methods to investigate the stereochemistry of a molecule is enantioselective high-performance liquid chromatography hyphenated to electronic circular dichroism (HPLC-CD). Application of enantioselective HPLC allows the separation of the enantiomeric fractions, than the sign of the ECD signal allows the AC determination of each fraction because two enantiomers are positively characterized by their fully opposite CD spectra. Combination of HPLC-CD with quantum mechanical calculation finally provides stereochemical characterization of chiral compounds.[15]

1.10 CD spectroscopy

Interaction between circularly polarized light with optical active compound cause formation of elliptically polarized light. This effect is called circular dichroism (CD). Circular dichroism can be observed only for chiral molecules or for ligand-host binding complexes if at least one of the interacting species is chiral.[16]

Linearly polarized light is composed of two circularly polarized components, one left circular and the other right circular.[16]

CD is a method based on the differential absorption of left and right circularly polarized light (Figure 5) by an optically active substance in the region of its absorption band. Differential absorption of the two circular components will lead to that of the linearly polarized light becomes elliptical with the orientation of the major axis of the ellipse defining the optical rotation. Chromophores are the parts of compound which are responsible for absorption.[16]

In chiral molecules, the movement of excited electrons, which is caused by interaction between circularly polarized light and chiral compound, is helical. The charge displacement can be left handed or right handed depending on the absolute configuration of atoms around the chromophore environment. Circularly polarized light exhibits preferential interactions: left handed charge displacement interacts preferentially with right circularly polarized light and right handed charge displacement with left circularly polarized light.[17-20]

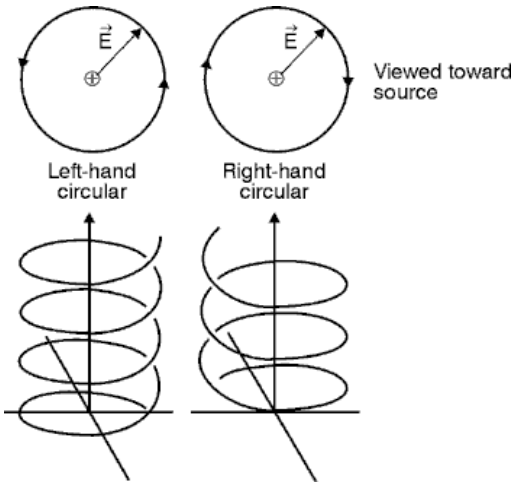


Figure 5. Schematic representation of left and right circularly polarized light. [21]

Linearly polarized light is divided by a modulator into two circularly polarized components – left and right. CD spectrophotometer detects both of these components separately. The differential absorption of circularly light is expressed by Beer-Lambert Law:

$$\Delta A = A_L - A_R = (\epsilon_L - \epsilon_R)cl = \Delta\epsilon cl \qquad \text{(Eq. 4)}$$

where ΔA is the differential absorbance between left circularly polarized (A_L) and right circularly polarized light (A_R), $\Delta\epsilon$ is the differential molar extinction coefficient, c is the concentration in moles per litre, and l is the cell path length in cm.[17]

Enantiomers will show CD spectra with identical profiles but with exactly opposite sign; consequently, a racemate will show no CD spectrum, because it is an equal mixture of enantiomers (Figure 6).

Defining whether the molecule is in *R* or *S* configuration can be determined from the CD spectra by comparison with quantum mechanical calculations or by comparison with the experimental CD spectra of similar structures.[16]

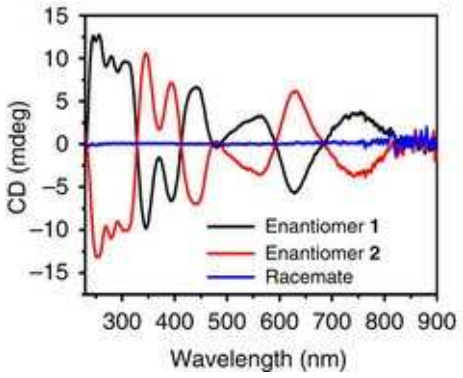


Figure 6. An example of CD spectra for a racemate and for its enantiomers (taken from ref.[22]).

1.11 The CD spectrometer

Figure 7 shows a schematic representation of a circular dichroism spectrometer. A xenon lamp produces white, non-polarized light, which passes through the monochromator and becomes monochromatic. A linear polarizer filters the monochromatic beam which becomes also linearly polarized. Then, monochromatic and linearly polarized light passes through a polarization modulator, which induces periodic changes between left circular light through elliptical, unchanged linear to elliptical and right circular light. The λ-drive changes the wavelength of light incident on the sample and ensures the polarization modulator works at the condition appropriate for the selected wavelength.[16, 18, 19] The beam passes through an optically active (chiral) sample. The sample shows the preferential absorption during one of the periodic variation and it is detected by a photomultiplier detector. Successive detection at various wavelengths leads to the generation of the full CD spectrum.[23]

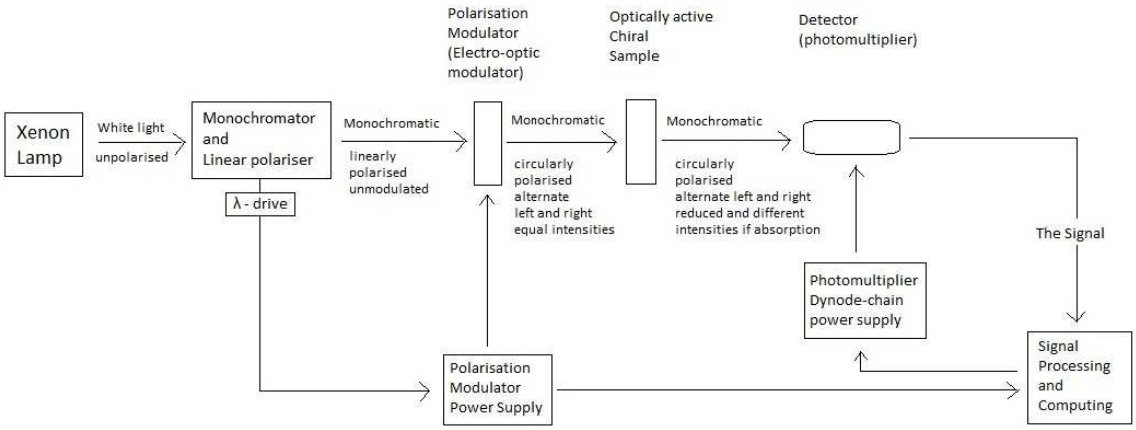


Figure 7. Diagram of the instrument setup of a CD spectrometer (taken from ref.[17]).

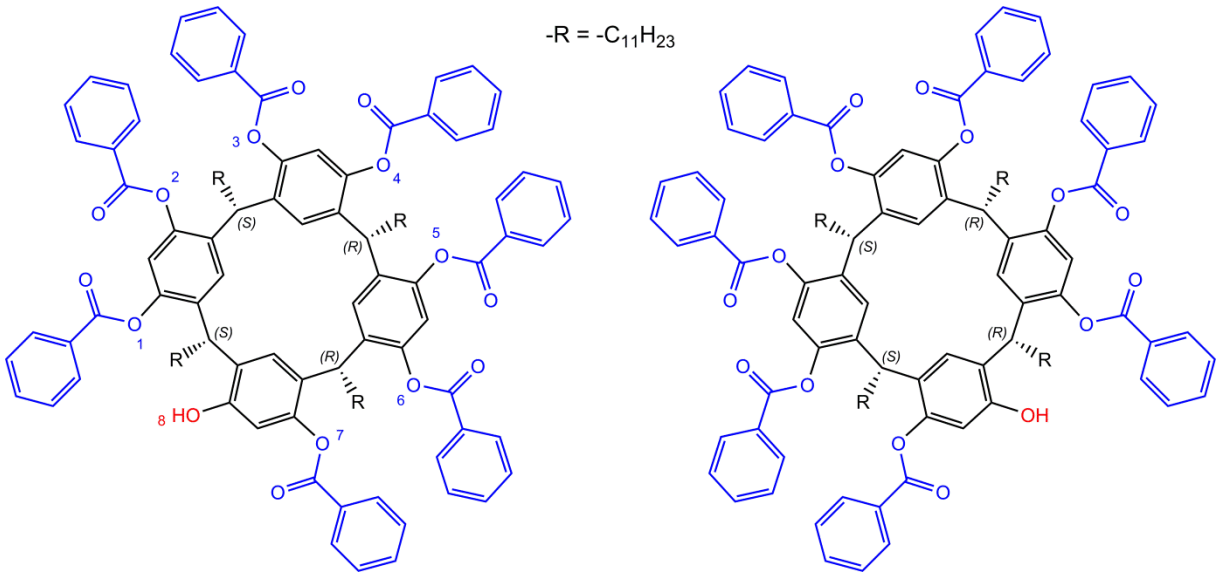
1.12 Resorcinarenes

Resorcinarenes [24, 25] are a type of calixarenes with a high potential to be used in a lot of different sectors. Structures of resorcinarenes are composed by macrocyclic circle prepared by condensation of resorcinol and an aldehyde in acidic environment. The first mention of resorcinarenes was in 1872 when Adolf von Baeyer reported that the condensation reaction between resorcinol and benzaldehyde in a concentrated sulfuric acid solution gave a red-colored product that turned to violet in alkaline solution. These macrocycles have opened access to promising supramolecular systems and they have been extensively studied by several scientific groups.

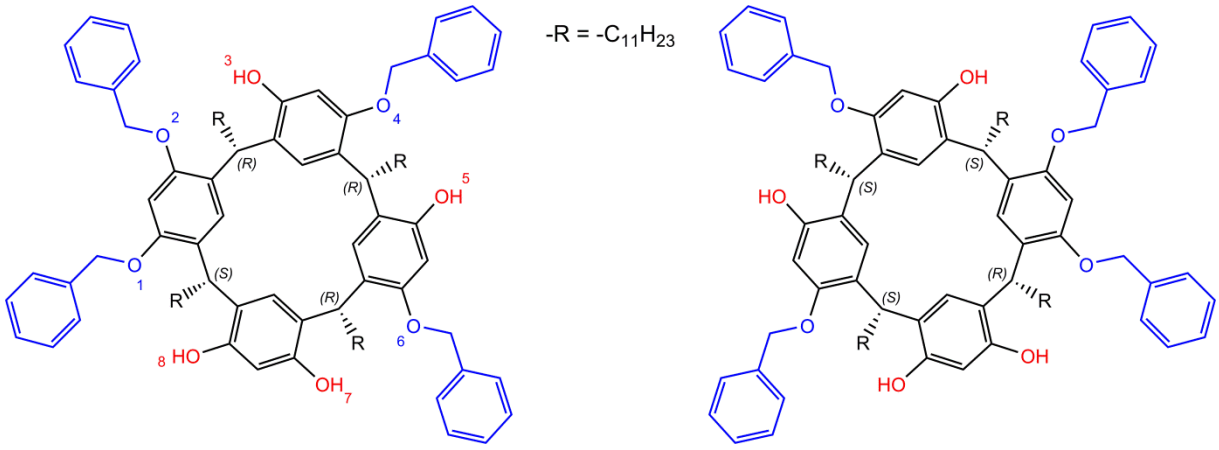
The structures of resorcinarenes in general are analogous to cyclic tetrameric structures frequently encountered in nature (porphyrins).

The nomenclature resorcin[4]arene is close to calix[4]arene and [4] represents the number of repeating units in the ring.

My work is focused on determining the stereochemistry of resorcin[4]arenes, which have been synthesized by weak base promoted *O*-alkylation of *C*-undecylresorcin[4]arene. The resorcin[4]arenes 1,2,3,4,5,6,7-*O*-heptabenzoyl-*C*-undecylresorcin[4]arene with trivial name ***7e*** and 1,2,4,6-*O*-tetrabenzyl-*C*-undecylresorcin[4]arene with trivial name ***5a*** are chiral and composed of two enantiomers (Figure 8). The objective of my work was to get separations of these enantiomers and collect one of the enantiomers for further modification and characterization.



A



B

Figure 8. Enantiomers of the analyzed compounds. A: ***7e***; B: ***5a***.

2 Experimental part

2.1 Aims of the project

The objective of my work was to obtain a separation of compound ***7e*** and ***5a***, which are part of a series of resorcin[4]arenes. These compound have been synthesized by weak base promoted *O*-alkylation of *C*-undecylresorcin[4]arenes. Compounds are chiral and composed of two enantiomers. Their use as building blocks for the preparation of supramolecular hosts requires their separation and subsequent characterization of their chirality.

For enantioseparation and further characterization of compounds was used enantioselective HPLC hyphenated with electronic circular dichroism detection (HPLC-CD). Chiral stationary phases based on polysaccharides were used to provide adequate HPLC enantioselectivity.

2.2 Materials

Compounds ***7e*** (1,2,3,4,5,6,7-*O*-heptabenzoyl-*C*-undecylresorcin[4]arene) and ***5a*** (1,2,4,6-*O*-tetrabenzyl-*C*-undecylresorcin[4]arene) were kindly provided by Prof. Placido Neri's research group (Department of Chemistry and Biology, University of Salerno, Italy). HPLC-grade solvent *n*-hexane 96% was purchased from Scharlab S.L. (Spain), while HPLC-grade solvents 2-propanol, methanol and ethanol were purchased from Sigma-Aldrich (Milan, Italy).

2.3 Instrumentation

The enantioselective HPLC method was carried out on a chromatographic system consisting of a Jasco PU-980 pump, a MD-910 UV/VIS DAD detector, a LG-2080-02 ternary gradient unit, a DG-2080-53 degasser, a Jones 7955 column chiller, and a Rheodyne 7725i syringe loading injector equipped with 20 µL and 100 µL sample loops. The UV and CD spectra were measured in the 350-215 nm spectral range on a Jasco (Tokyo, Japan) J-810 spectropolarimeter equipped with a 10 mm pathlength HPLC flow cell and a Rheodyne 7010 injector used as a three-way valve. 100 µL and 250 µL Hamilton syringes were used for injections.

The columns used for the analysis were:

- Phenomenex Lux Amylose-2 [amylose tris(5-chloro-2-methylphenylcarbamate) coated on 5 µm silica gel particles; 250x4.6 mm I.D.];
- Phenomenex Lux Cellulose-1 [cellulose tris(3,5-dimethylphenylcarbamate) coated on 5µm silica gel particles; 250x4.6 mm I.D.];
- Phenomenex Lux Cellulose-2 [cellulose tris(3-chloro-4-methylphenylcarbamate) coated on 5µm silica gel particles; 250x4.6 mm I.D.];
- Phenomenex Lux Cellulose-3 [cellulose tris(4-methylbenzoate) coated on 5µm silica gel particles; 250x4.6 mm I.D.];
- Phenomenex Lux Cellulose-4 [cellulose tris(4-chloro-3-methylphenylcarbamate) coated on 5µm silica gel particles; 250x4.6 mm I.D.];
- Daicel Chiralcel OJ [cellulose tris(4-methylbenzoate) coated on 10µm silica gel particles; 250x4.6 mm I.D.];
- Daicel Chiralpak AD [amylose tris(3,5-dimethylphenylcarbamate) coated on 10µm silica gel particles; 250x4.6 mm I.D.];

- Daicel Chiralcel OD-H [cellulose tris(3,5-dimethylphenylcarbamate) coated on 5μm silica gel particles; 250x4.6 mm I.D.];
- Merck KGaA Hibar RT Pirkle (S,S)-Whelk-O 1; coated on 5μm silica gel particles; 250x4.6 mm I.D.

2.4 Sample preparation

The samples of compounds ***7e*** and ***5a*** were prepared by dissolving in *n*-hexane. As a start, the volume and concentration of injected samples for the optimization of chromatographic conditions were set at 20 μL and 0.25 mg/mL of racemic mixture in *n*-hexane. Higher concentration was not needed for finding appropriate ways of separation. The volume and concentration of samples for preparative injections of ***7e*** were set at 100 μL and 1 mg/mL of racemic mixture in *n*-hexane.

2.5 Analysis on compound *7e*

According to previous analysis of ***7e***, HPLC column Chiralpak AD was used for the enantiomeric separation in normal phase conditions (mobile phase: *n*-hexane/2-propanol 98:2, v/v; flow rate: 1mL/min). It was decided to try other columns suitable for enantiomeric analysis with similar proportions of mobile phase and the same flow rate, in order to get a better resolution.

Since previous testing was performed using *n*-hexane/2-propanol mixtures ranging from 97:3 to 99:1 (v/v) and flow rates ranging from 1 to 2 mL/min, the starting point for new analyses with the new columns was always set to a mobile phase ratio of 95:5 (*n*-hexane/2-propanol). According to the following results was edited the ratio of the mobile phase.

The first analysis revealed that the separation is very sensitive to the amount of 2-propanol used in the mobile phase.

Use of pure hexane and pure 2-propanol and their subsequent mixing in the HPLC mixing unit is not able to ensure a homogeneous environment for the analysis. Due to this fact, the mobile phase must be prepared in advance in exact proportions. In our case, a 90:10 v/v mixture of *n*-hexane and 2-propanol was prepared and mixed with pure *n*-hexane by the HPLC mixing unit, in order to get a more changeable condition for the analyses.

With larger amounts of hexane, better separation was observed, but with prolongation of the retention times. Increasing levels of 2-propanol caused a worse separation of peaks which is undesirable, despite of the reduction of retention times. To arrive at effective results, we increased the proportion of hexane gradually.

For columns which had poor resolution of peaks we tried to modify the conditions by increasing the proportion of hexane and increasing the flow to 2 mL/min, if needed to reduce the time of analysis.

2.6 Analysis on compound *5a*

For analysis of ***5a*** it had no previous results, therefore we followed the same protocol used for compound ***7e***. The starting point was performed using *n*-hexane/2-propanol mixtures 97:3 (v/v) and flow rate 1 mL/min in column Lux Cellulose-1.

Initial analyses revealed that the conditions for compound ***7e*** are different than for compound ***5a***, because of the polarity of compound ***5a***. Despite of the fact, that compound ***7e*** and ***5a*** are the same series of resorcin[4]arenes, the higher polarity of compound ***5a*** made 2-propanol inappropriate for the resolutions of peaks. It was tried to replace 2-propanol by ethanol or methanol, in order to get resolution.

Methanol is generally non-miscible with *n*-hexane, but it is possible to mix them in some proportions: for methanol, 5% is the highest proportion, at which it can be mixed with *n*-hexane. Due to this fact, it could use *n*-hexane/methanol 95:5 and gradually increase the proportion of *n*-hexane. Moreover, adding formic or acetic acid can improve the resolution.

3 Results

3.1 Analysis on compound *7e*

Table 2. Enantioresolution of compound ***7e*** on the Chiralcel OJ column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Chiralcel OJ	*n*-hexane/2-propanol	95:5	1.0	3.536	4.080	1.154	---
Chiralcel OJ	*n*-hexane/2-propanol	98:2	1.0	8.208	10.844	1.321	1.018

Table 3. Enantioresolution of compound ***7e*** on the Lux Cellulose-2 column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Lux Cellulose-2	*n*-hexane/2-propanol	95:5	1.0	1.227	1.577	1.285	---
Lux Cellulose-2	*n*-hexane/2-propanol	98:2	1.0	4.166	5.813	1.395	---

Table 4. Enantioresolution of compound ***7e*** on the Chiralpak AD column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Chiralpak AD	*n*-hexane/2-propanol	95:5	1.0	0.454	---	---	---
Chiralpak AD	*n*-hexane/2-propanol	98:2	1.0	1.633	1.758	1.077	---
Chiralpak AD	*n*-hexane/2-propanol/ formic acid	98:2:0.1	1.0	1.971	2.119	1.075	---
Chiralpak AD	*n*-hexane/2-propanol	99:1	1.0	5.754	6.771	1.177	---
Chiralpak AD	*n*-hexane/2-propanol	99:1	2.0	5.253	5.767	1.098	---

Table 5. Enantioresolution of compound ***7e*** on the Lux Cellulose-3 column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Lux Cellulose-3	*n*-hexane/2-propanol	95:5	1.0	3.640	4.328	1.189	1.163
Lux Cellulose-3	*n*-hexane/2-propanol	96:4	1.0	4.353	5.267	1.210	1.257
Lux Cellulose-3	*n*-hexane/2-propanol	97:3	1.0	6.015	7.508	1.248	1.387
Lux Cellulose-3	*n*-hexane/2-propanol	98:2	1.0	9.403	12.163	1.294	1.513
Lux Cellulose-3	*n*-hexane/2-propanol	98:2	2.0	7.013	9.077	1.294	1.580

Table 6. Enantioresolution of compound ***7e*** on the Lux Cellulose-4 column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Lux Cellulose-4	*n*-hexane/2-propanol	95:5	1.0	1.578	1.910	1.210	---
Lux Cellulose-4	*n*-hexane/2-propanol	98:2	1.0	6.612	8.148	1.232	---

Table 7. Enantioresolution of compound ***7e*** on the Lux Cellulose-1 column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Lux Cellulose-1	*n*-hexane/2-propanol	95:5	1.0	1.512	2.096	1.386	1.388
Lux Cellulose-1	*n*-hexane/2-propanol	96:4	1.0	1.805	2.523	1.397	1.444
Lux Cellulose-1	*n*-hexane/2-propanol	97:3	1.0	2.724	3.821	1.403	1.483
Lux Cellulose-1	*n*-hexane/2-propanol	98:2	1.0	4.957	7.026	1.417	1.525

Table 8. Enantioresolution of compound ***7e*** on the Chiralcel OD-H column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Chiralcel OD-H	*n*-hexane/2-propanol	95:5	1.0	0.581	0.969	1.668	1.082
Chiralcel OD-H	*n*-hexane/2-propanol	97:3	1.0	0.938	1.694	1.806	1.371
Chiralcel OD-H	*n*-hexane/2-propanol	98:2	1.0	1.530	2.950	1.929	1.588

Table 9. Enantioresolution of compound ***7e*** on the Hibar column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Hibar	*n*-hexane/2-propanol	95:5	1.0	---	---	---	---
Hibar	*n*-hexane/2-propanol	80:20	1.0	7.525	---	---	---
Hibar	*n*-hexane/2-propanol	80:20	1.0	6.239	---	---	---

Table 10. Enantioresolution of compound ***7e*** on the Lux Amylose-2 column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Lux Amylose-2	*n*-hexane/2-propanol	95:5	1.0	---	---	---	---
Lux Amylose-2	*n*-hexane/2-propanol	98:2	1.0	---	---	---	---
Lux Amylose-2	*n*-hexane/2-propanol	99:1	1.0	---	---	---	---
Lux Amylose-2	*n*-hexane/2-propanol	100:0	1.0	---	---	---	---

According to the results we came to the conclusion, that the best column for the collection is Lux Cellulose-1. Despite of the fact that column Chiralcel OD-H showed a little better separation (Table 8) than Lux Cellulose-1 (Table 7), since the retention time was significantly shorter on the Chiralcel OD-H (Figure 9). The separation and subsequent collection of the peak would have been more difficult to achieve, so it was decided to use a newer column with a longer retention time, but with higher efficiency.

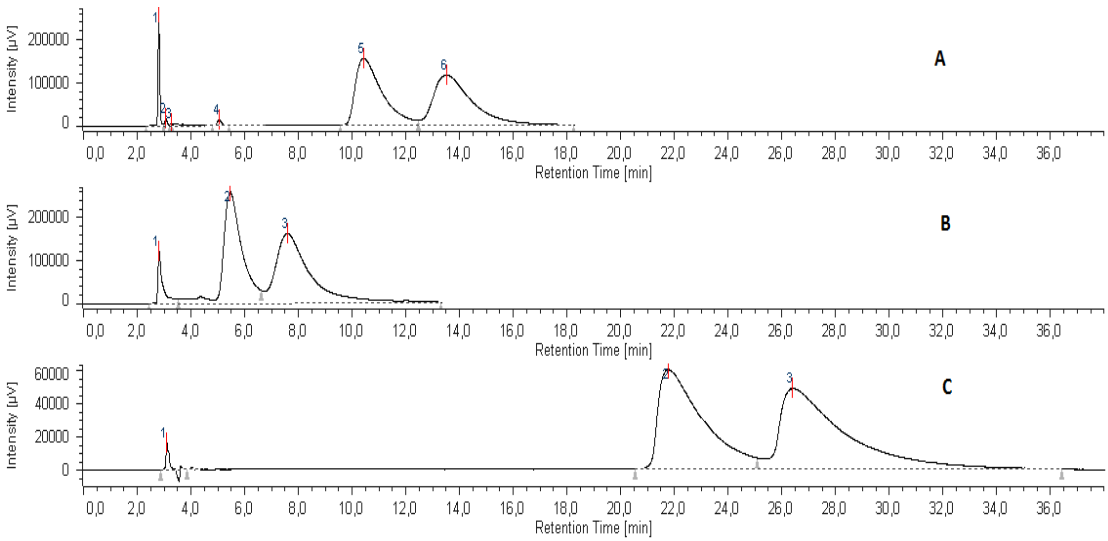


Figure 9. Chromatograms for enantioselective HPLC analysis of compound ***7e*** by A: Lux Cellulose-1, B: Chiralcel OD-H, C: Lux Cellulose-3 in the same conditions (mobile phase: *n*-hexane/2-propanol 97:3, v/v; flow rate: 1mL/min; λ = 230 nm).

For sample collection, it was used the column Lux Cellulose-1 under the following conditions: mobile phase *n*-hexane/2-propanol 97:3 (v/v), flow rate 1mL/min, λ=230nm (Figure 10). Concentration 1 mg/mL in *n*-hexane was chosen for preparative injections.

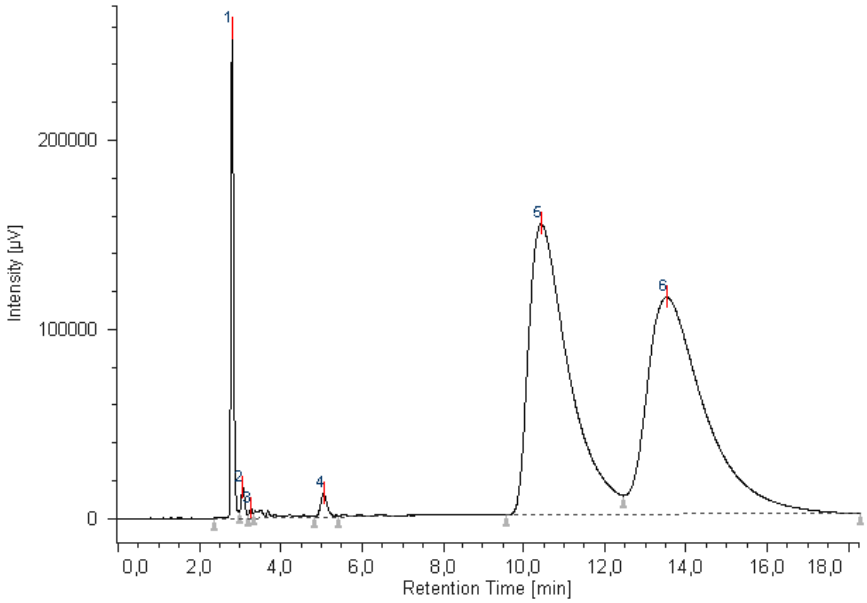


Figure 10. Chromatogram for the enantioselective HPLC analysis of compound ***7e*** on Lux Cellulose-1 under optimized conditions (mobile phase: *n*-hexane/2-propanol 97:3, v/v; flow rate: 1 mL/min; λ = 230 nm).

Every day I had to prepare a new sample for injection to avoid possible degradation of it and subsequently I injected 100µL to separate the enantiomers and collect the first eluted fraction. The collection was made manually into the vial. Around 16 injections were performed every day of collection (Figure 11). The collected amount of pure enantiomer had to be evaporated by nitrogen to avoid a potential degradation of the sample caused by the amount of 2-propanol inside the vial.

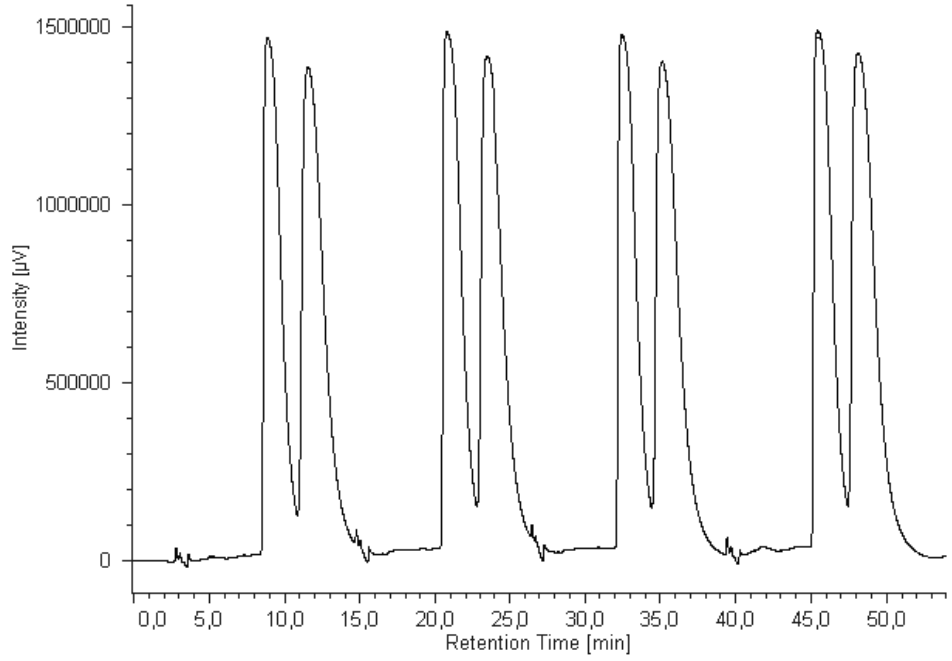


Figure 11. Chromatogram for the preparative collection of compound ***7e*** on Lux Cellulose-1 under optimized conditions.

The first week of collection brought us about 2-3 mg of the desired enantiomer, which was quantified by a calibration curve built with increasing concentration of racemic ***7e***. Thanks to the calibration curve we got a clue about the quality, quantity and purity of the collected enantiomer. We collected the sample for 3 weeks and got 8.12 mg with enantiomeric excess > 99.9% (Figure 12) of the first enantiomer of compound ***7e***.

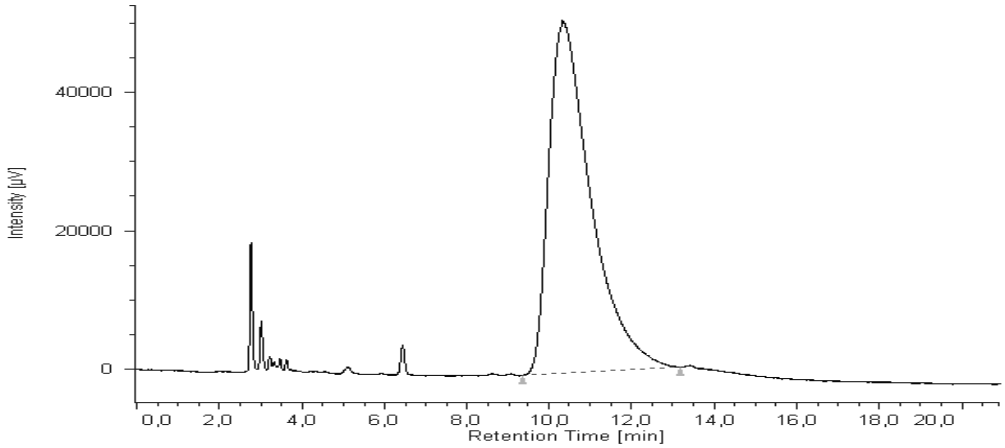


Figure 12. Chromatogram for the collected enantiomeric fraction of compound ***7e*** on Lux Cellulose-1 under optimized conditions.

The optimized conditions were also used to perform a HPLC-CD analysis on compound ***7e*** and obtain the ECD spectra of its enantiomers, shown in Figure 13. The profiles of the spectra are, as expected for enantiomers, identical and of opposite sign.

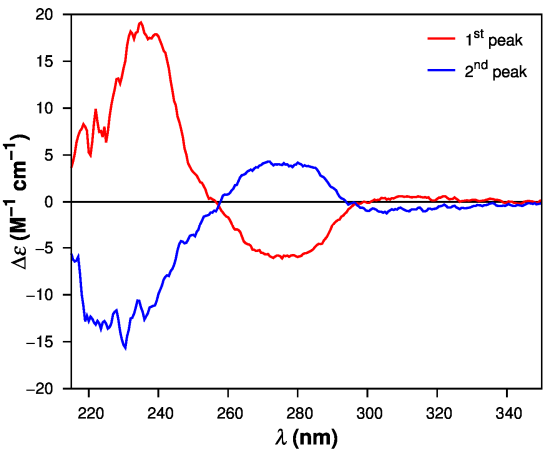


Figure 13. ECD spectra of the enantiomeric fractions of compound ***7e*** obtained by HPLC-CD analysis on Lux Cellulose-1 under optimized conditions.

3.2 Analysis on 5a

Table 11. Enantioresolution of compound ***5a*** on the Lux Cellulose-1 column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Lux Cellulose-1	*n*-hexane/2-propanol	97:3	1.0	---	---	---	---
Lux Cellulose-1	*n*-hexane/2-propanol	80:20	1.0	---	---	---	---

Table 12. Enantioresolution of compound ***5a*** on the Lux Cellulose-4 column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Lux Cellulose-4	*n*-hexane/2-propanol	80:20	1.0	---	---	---	---

Table 13. Enantioresolution of compound ***5a*** on the Lux Cellulose-3 column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Lux Cellulose-3	*n*-hexane/2-propanol	80:20	1.0	---	---	---	---

Table 14. Enantioresolution of compound ***5a*** on the Chiralpak AD column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Chiralpak AD	*n*-hexane/2-propanol	80:20	1.0	---	---	---	---
Chiralpak AD	*n*-hexane/2-propanol	90:10	1.0	---	---	---	---

Table 15. Enantioresolution of compound ***5a*** on the Lux Cellulose-2 column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Lux Cellulose-2	*n*-hexane/2-propanol	90:10	1.0	---	---	---	---
Lux Cellulose-2	*n*-hexane/methanol	95:5	1.0	---	---	---	---

Table 16. Enantioresolution of compound ***5a*** on the Lux Amylose-2 column.

Column	Mobile phase	Composition (v/v)	Flow rate (mL/min)	k_1	k_2	α	R_s
Lux Amylose-2	*n*-hexane/2-propanol	80:20	1.0	1.728	2.039	1.180	---
Lux Amylose-2	*n*-hexane/2-propanol	85:15	1.0	3.578	4.214	1.178	---
Lux Amylose-2	*n*-hexane/ethanol	90:10	1.0	1.429	1.783	1.248	0.707
Lux Amylose-2	*n*-hexane/methanol	95:5	1.0	0.857	0.952	1.110	1.000
Lux Amylose-2	*n*-hexane/methanol	96:4	1.0	1.501	1.792	1.194	1.522
Lux Amylose-2	*n*-hexane/methanol	96.5:3.5	1.0	2.295	2.779	1.211	1.447
Lux Amylose-2	*n*-hexane/methanol/ acetic acid	96:4:0.1	1.0	1.114	1.354	1.216	1.793

According to the results for ***5a*** we came to the conclusion that the best column for separation is Lux Amylose-2 under conditions *n*-hexane/methanol 96:4, flow rate 1 mL/min (Figure 14); the addition of acetic acid further improved the resolution (Figure 15). Enantiomeric resolution was observed only in the column Lux Amylose-2. We had to use more polar conditions with ethanol and methanol instead of 2-propanol, because compound ***5a*** is more polar than compound ***7e*** and using 2-propanol was inappropriate. The other columns observed no separation (Figure 16). Instead of 2- propanol it was decided to use ethanol first, which is more polar than 2-propanol, but even in this case, the sufficient peaks separation did not appear (Figure 17). For improving separation, we successfully employed methanol.

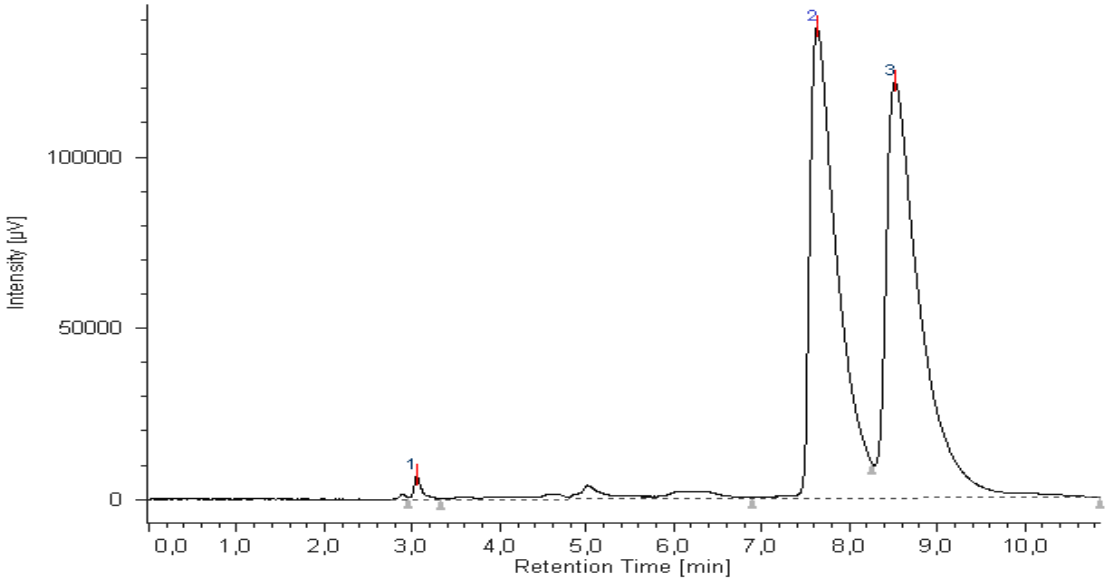


Figure 14. Chromatogram for enantioselective HPLC analysis of compound ***5a*** on Lux Amylose-2 under conditions *n*-hexane/methanol 96:4 (v/v), flow rate 1 mL/min.

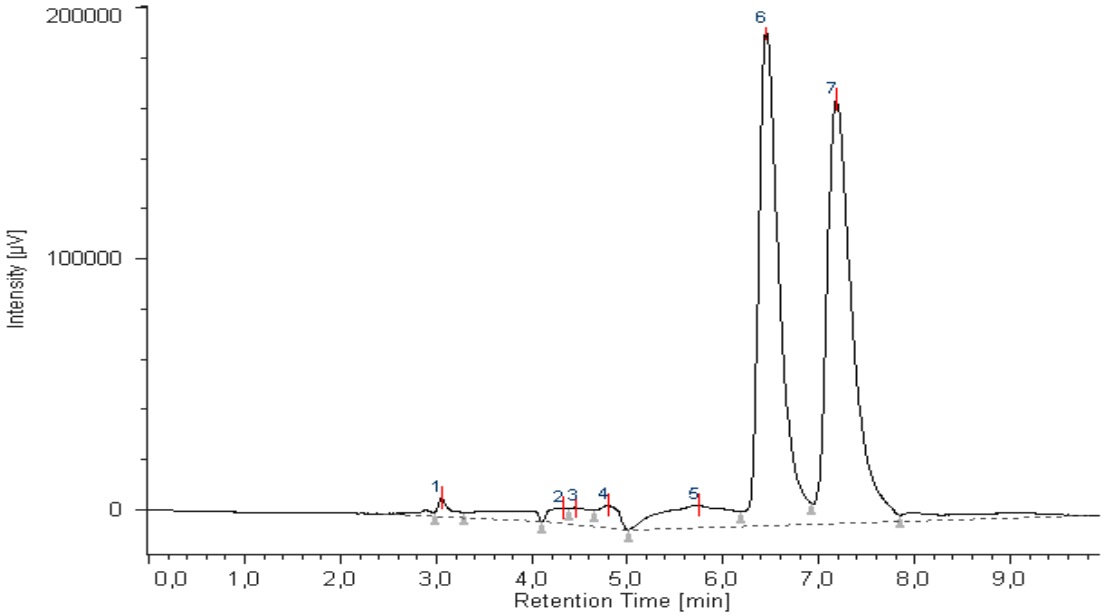


Figure 15. Chromatogram for enantioselective HPLC analysis of compound ***5a*** on Lux Amylose-2 under conditions *n*-hexane/methanol/acetic acid 96:4:0.1 (v/v/v), flow rate 1mL/min.

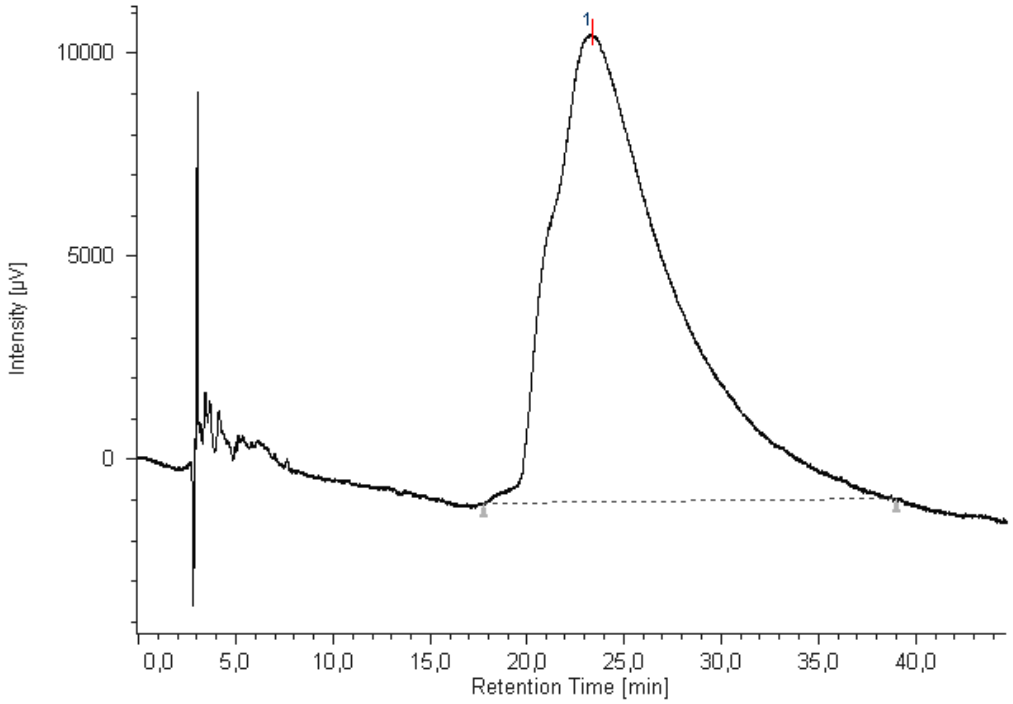


Figure 16. Chromatogram for enantiomeric HPLC analysis for compound ***5a*** by Lux Cellulose-1 under conditions *n*-hexane/2-propanol 80:20 (v/v), flow rate 1 mL/min.

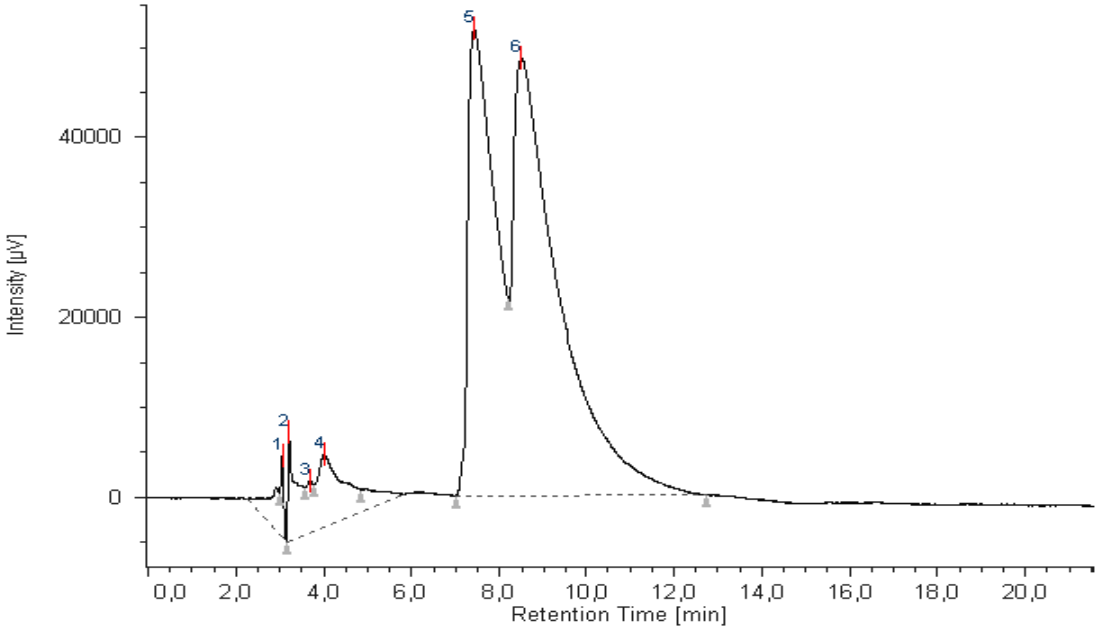


Figure 17. Chromatogram for enantioselective HPLC analysis for compound ***5a*** by Lux Amylose-2 under conditions *n*-hexane/ethanol 90:10 (v/v), flow rate 1 mL/min.

Similarly to what was done with compound ***7e***, a HPLC-CD analysis on compound ***5a*** was performed under optimized conditions with the aim of measuring the ECD spectra of its enantiomers, shown in Figure 18. The profiles of the spectra are, once again, identical and of opposite sign, confirming that the two fractions are in enantiomeric relationship.

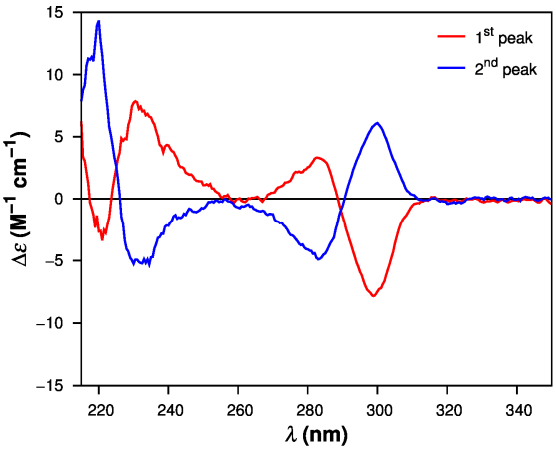


Figure 18. ECD spectra of the enantiomeric fractions of compound ***5a*** obtained by HPLC-CD analysis on Lux Amylose-2 under optimized conditions.

4 Discussion

The aim of my project was to get enantioseparation of compounds ***7e*** and ***5a*** and to collect one of the enantiomers of ***7e*** for further modification and characterization. These compounds are chiral and composed of two enantiomers; their use as building blocks for the preparation of supramolecular hosts requires their separation and subsequent characterization of their chirality.

There are possibilities to use different analytical separation methods suitable for the separation of chiral molecules. The main technique used for the enantioseparation of these molecules was enantioselective HPLC based on polysaccharides stationary phases. By hyphenating HPLC with electronic circular dichroism (HPLC-CD), the successful enantioseparation was confirmed and the ECD spectra of pure enantiomers were measured.

Syntheses of ***7e*** and ***5a*** were performed by Prof. Placido Neri's research group (Department of Chemistry and Biology, University of Salerno, Italy). The first enantioseparation of ***7e*** revealed the conditions for separation: HPLC column Chiralpak AD in normal phase conditions (mobile phase: *n*-hexane/2-propanol 98:2, v/v; flow rate: 1mL/min). It was decided to try other columns suitable for enantiomeric analysis with similar proportions of mobile phase and the same flow rate, in order to get a better resolution.

The best column for the separation of the enantiomers of ***7e*** was Chiralcel OD-H. Despite of the result it was decided to use column Lux Cellulose-1 for the collection of the first-eluted enantiomer, under similar conditions (mobile phase: *n*-hexane/2-propanol 97:3, v/v; flow rate: 1mL/min; λ = 230 nm).

Lux Cellulose-1 was a newer column with higher efficiency than Chiralcel OD-H, and longer retention times on the Lux Cellulose-1 allowed better preparative conditions to obtain a sample of higher purity.

The possibility of collecting the sample using the Chiralcel OD-H column and making comparison with the results achieved from the collection with Lux Cellulose-1 was considered, but the resulting yields (collection of 8.12 mg of the first enantiomer of compound ***7e*** with enantiomeric excess > 99.9%) were fully satisfactory and so there was no need to achieve higher values of purity.

The analyses revealed that the sample and separation is very sensitive to the amount of 2-propanol: every day of collection, a new sample had to be prepared for injection in order to avoid possible degradation and the collected amount of pure enantiomer had to be evaporated by nitrogen to avoid a potential degradation of the sample caused by the amount of 2-propanol inside the vial. The developed method for separation and collection of compound ***7e*** can be safely and usefully applied for further analyses and collections of compound ***7e***.

No previous results were available for the analysis of ***5a***, therefore the same protocol for compound ***7e*** was followed. However, initial analyses revealed that the conditions for compound ***7e*** are different than for compound ***5a***, because of the polarity of compound ***5a***. The higher polarity made 2-propanol inappropriate for the resolutions of peaks. Nevertheless a suitable choice for replacement was the use of ethanol or methanol. Moreover, adding formic or acetic acid can improve the resolution. There is also potential possibility to use other mobile phases, additives or methods suitable for enantioseparation.

The obtained ECD spectra for the enantiomers of ***7e*** and ***5a*** will be used for the characterization of their chirality by comparison with quantum mechanical calculations, which are part of the future development of this project. The structure of compound ***7e***, however, is too large for quantum mechanical calculations.

A possible solution to solve this problem is to simplify its structure by a chemical modification able to preserve information on the chirality of the parent compound, and subsequently perform the quantum mechanical calculations on the simplified structure. This can be achieved by methylation of the single free hydroxyl group and subsequent removal of the 7 benzoyl groups: the product of this structural simplification is *O*-methyl-*C*-undecylresorcin[4]arene (Figure 19), labeled ***8c***, whose racemate was synthesized by Prof. Placido Neri's research group at the the Department of Chemistry and Biology, University of Salerno, Italy. Compound ***8c*** is much more polar than compounds ***7e*** or ***5a.*** Because of the polarity of ***8c***, initial tests showed that enantioresolution could not be achieved under normal phase conditions or in polar organic mode. Future efforts on this project will focus on finding the optimal condition for the enantioseparation of compound ***8c***, with the final aim of determining which of the two enantiomers of **8c** is obtained by reaction of the pure ***7e*** enantiomer collected during my thesis and relate the stereochemistry of ***7e*** to the stereochemistry of ***8c***.

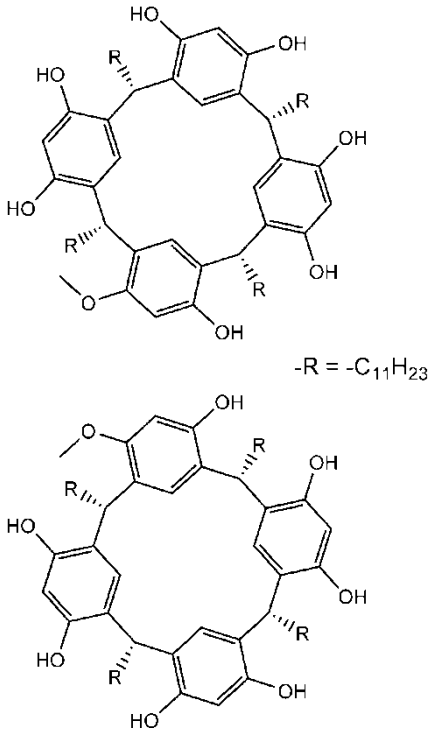


Figure 19. Enantiomers of compound ***8c***.

Compounds ***7e***, ***5a*** and ***8c*** are new molecules with no references on analytical protocol for their stereochemical characterization, for this reason my thesis can be used for further research of resorcin[4]arenes.

The project was presented by Daniele Tedesco, Ph.D. at a scientific conference - Recent developments in pharmaceutical analysis 2015 (RDPA 2015) in Perugia, June 28 – July 1, 2015. The poster gained the Best Poster Award 1st Prize at the RDPA 2015 conference. The poster is attached in the annex.

5 Conclusion

In this project, the conditions for the enantioseparation of chiral compounds ***7e*** and ***5a*** by enantioselective HPLC were optimized. It was concluded that the best columns for the separation of the enantiomers ***7e*** were Chiralcel OD-H and Lux Cellulose-1. Lux Cellulose-1 seemed to have a higher efficiency than Chiralcel OD-H, for this reason Lux Cellulose-1 was chosen for the HPLC-CD analysis and for the preparative collection of the first-eluted enantiomer. For the collection we decided to use a mobile phase composed by *n*-hexane and 2-propanol in 97:3 (v/v) proportion and a flow rate of 1 mL/min. The concentration for injection was 1 mg/mL in the amount of 100 µL. After 3 weeks we obtained 8.12 mg with enantiomeric excess > 99.9% of the first enantiomer of compound ***7e***. After HPLC-CD analysis, the resulting mirror-image ECD spectra of the fractions of **7e** were in good enantiomeric relationship. On the other hand, the best chromatographic conditions for the enantioseparation of compound ***5a*** were obtained on Lux Amylose-2 column in conditions *n*-hexane/methanol/acetic acid 96:4:0.1 and flow rate of 1 mL/min; also in this case, the ECD spectra of the fractions obtained by HPLC-CD analysis were in mirror-image relationship.

List of figures

List of tables

List of annex

Presented poster from a scientific conference: RDPA 2015 - Recent developments in pharmaceutical analysis, Perugia June 28 – July 1, 2015

Development of stopped-flow enantioselective HPLC-CD methods:

Towards the stereochemical characterization of C-undecylresorcin[4]arenes

Abbreviations

A	absorbance
AC	absolute configuration
A_L	absorbance of left circularly polarized light
A_R	absorbance of right circularly polarized light
α	selectivity
c	concentration
CD	circular dichroism
CSPs	chiral stationary phases
ECD	electronic circular dichroism
ϵ	molar extinction coefficient
HPLC	high performance liquid chromatography
HPLC-CD	high performance liquid chromatography hyphenated to electronic circular dichroism
κ	capacity factor
l	cell path length
RDPA	Recent developments in pharmaceutical analysis
RP	reversed phased
Rs	resolution
t_r	retention time
t_0	void time
UV/VIS	ultraviolet/visible light
w_1	width of the first peak
w_2	width of the second peak

References

1. VSCHT. Optické isomery - enantiomery 2008 [cited 2015 5.5.].
2. Douša, M. *http://www.hplc.cz/Chiral/index.htm*. 19.2.2015 [cited 2015 15.5.].
3. Zhang, Y., et al., *Enantioselective chromatography in drug discovery.* Drug Discovery Today, 2005. **10**(8): p. 571-577.
4. Lämmerhofer, M., *Chiral recognition by enantioselective liquid chromatography: Mechanisms and modern chiral stationary phases.* J. Chromatogr. A, 2010. **1217**(6): p. 814-856.
5. Clark, J. *Chemguide*. High performance liquid chromatography 2007 April 2015 [cited 2015 5.5.]; Available from: http://www.chemguide.co.uk/analysis/chromatography/hplc.html.
6. Larsen, D. *Analytical chemistry*. High performance liquid chromatography [cited 2015 5.5.]; Available from: http://chemwiki.ucdavis.edu/Analytical_Chemistry/Instrumental_Analysis/Chromatography/High_performance_liquid_chromatography.
7. Lindsay, S. and J. Barnes, *High Performance Liquid Chromatography*1992: Wiley.
8. Klimeš, J., *Kontrola léčiv I.* 2. ed2008: Karolinum, Praha1, Ovocný trh 3. 149.
9. Tachibana, K. and A. Ohnishi, *Reversed-phase liquid chromatographic separation of enantiomers on polysaccharide type chiral stationary phases.* J. Chromatogr. A, 2001. **906**(1–2): p. 127-154.
10. Yashima, E., *Polysaccharide-based chiral stationary phases for high-performance liquid chromatographic enantioseparation.* J. Chromatogr. A, 2001. **906**(1–2): p. 105-125.
11. Okamoto, Y. and E. Yashima, *Polysaccharide Derivatives for Chromatographic Separation of Enantiomers.* Angewandte Chemie International Edition, 1998. **37**(8): p. 1020-1043.

12. Zhang, T., et al., *Cellulose 3,5-dimethylphenylcarbamate immobilized on silica: A new chiral stationary phase for the analysis of enantiomers.* Anal. Chim. Acta, 2006. **557**(1–2): p. 221-228.
13. Zhang, T., et al., *Cellulose tris(3,5-dichlorophenylcarbamate) immobilised on silica: A novel chiral stationary phase for resolution of enantiomers.* J. Pharm. Biomed. Anal., 2008. **46**(5): p. 882-891.
14. Francotte, E.R., *Enantioselective chromatography as a powerful alternative for the preparation of drug enantiomers.* J. Chromatogr. A, 2001. **906**(1–2): p. 379-397.
15. Bertucci, C. and D. Tedesco, *Advantages of electronic circular dichroism detection for the stereochemical analysis and characterization of drugs and natural products by liquid chromatography.* J. Chromatogr. A, 2012. **1269**: p. 69-81.
16. Harding, S.E. and B.Z. Chowdhry, *Protein-ligand Interactions, Structure and Spectroscopy: A Practical Approach*. Circular dichroism2001: Oxford University Press. 43.
17. Harding, S.E. and B.Z. Chowdhry, *Protein-ligand Interactions, Structure and Spectroscopy: A Practical Approach*2001: Oxford University Press. 123-166.
18. Brittain, H.G., *Analytical instrumentation handbook*. 2 ed. Techniques of chiroptical spectroscopy, ed. G.W. Ewing1997: Marcel Dekker, Inc. New York.
19. W.Curtis Johnson, J., *Circular dichroism and the Conformational Analysis of Biomolecules*, G.D. Fasman, Editor 1996, Plenum Press, New York. p. 635 -651.
20. Drake, A.F., *Methods in molecular biology, Vol 22: Microscopy, Optical Spectroscopy, and Macroscopic techniques*. Circular Dichroism, ed. B.M. C. Jones, A.H. Thomas. Vol. 16. 1994: Humana Press Inc. Totowa, NJ.
21. *Circularly polarized light*. [cited 2015 16.5.]; Available from: http://pixshark.com/circularly-polarized-light.htm.

22. Dolamic, I., et al., *First enantioseparation and circular dichroism spectra of Au38 clusters protected by achiral ligands.* Nat Commun, 2012. **3**: p. 798.
23. Jones, C., *Microscopy, Optical Spectroscopy, and Macroscopic Techniques*, in *Methods in molecular biology*1994, Humana Press. p. 251.
24. Timmerman, P., W. Verboom, and D.N. Reinhoudt, *Resorcinarenes.* Tetrahedron, 1996. **52**(8): p. 2663-2704.
25. F. Farina, C.T., C. Gaeta, P. Neri, *Regioselective O-Substitution of C-Undecylresorcin[4]arene.* ORGANIC LETTERS, 2011. **13**.